Control of Translation by the 5′- and 3′-Terminal Regions of the Dengue Virus Genome

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The genomic RNAs of flaviviruses such as dengue virus (DEN) have a 5′ m^7GpppN cap like those of cellular mRNAs but lack a 3′ poly(A) tail. We have studied the contributions to translational expression of 5′- and 3′-terminal regions of the DEN serotype 2 genome by using luciferase reporter mRNAs transfected into Vero cells. DCLD RNA contained the entire DEN 5′ and 3′ untranslated regions (UTRs), as well as the first 36 codons of the capsid coding region fused to the luciferase reporter gene. Capped DCLD RNA was as efficiently translated in Vero cells as capped GLGpA RNA, a reporter with UTRs from the highly expressed α-globin mRNA and a 72-residue poly(A) tail. Analogous reporter RNAs with regulatory sequences from West Nile and Sindbis viruses were also strongly expressed. Although capped DCLD RNA was expressed much more efficiently than its uncapped form, uncapped DCLD RNA was translated 6 to 12 times more efficiently than uncapped RNAs with UTRs from globin mRNA. The 5′ cap and DEN 3′ UTR were the main sources of the translational efficiency of DCLD RNA, and they acted synergistically in enhancing translation. The DEN 3′ UTR increased mRNA stability, although this effect was considerably weaker than the enhancement of translational efficiency. The DEN 3′ UTR thus has translational regulatory properties similar to those of a poly(A) tail. Its translation-enhancing effect was observed for RNAs with globin or DEN 5′ sequences, indicating no codependency between viral 5′ and 3′ sequences. Deletion studies showed that translational enhancement provided by the DEN 3′ UTR is attributable to the cumulative contributions of several conserved elements, as well as a nonconserved domain adjacent to the stop codon. One of the conserved elements was the conserved sequence (CS) CS1 that is complementary to cCS1 present in the 5′ end of the DEN polyprotein open reading frame. Complementarity between CS1 and cCS1 was not required for efficient translation.

Dengue virus (DEN) is a member of the mosquito-borne group of flaviviruses that includes yellow fever, Japanese encephalitis, and West Nile (WNV) viruses. It is the causative agent of millions of human infections annually in the tropical regions of the world (14). The virus has a vertebrate host range limited to humans and monkeys, but replication and amplification also occur in mosquito vectors, principally those of the *Aedes* genus (4).

The DEN genome is an 11-kb positive-strand RNA with a m^7GpppA cap at the 5′ end but lacking a poly(A) tail at the 3′ end (29). It encodes a single long polyprotein (see Fig. 1A) that is processed through the action of viral and host proteinases to generate 10 mature structural and nonstructural proteins. The maturation pathway, the cleavage sites, and the responsible proteinases have been well described (29). However, systematic studies of the roles of the untranslated regions (UTRs) of the viral RNA in the gene expression of DEN or other flaviviruses are lacking. The UTRs are expected to play an important early role during viral infection in coordinating viral gene expression and the onset of RNA replication.

Translation of the typical cellular mRNA relies on the recruitment of ribosomes by features in the UTRs at both ends of the RNA (11, 20, 31, 40, 41). The 5′ cap is recognized by the cytoplasmic cap binding protein eIF4E, which contacts initiation factor eIF4G. This large protein acts as a scaffold for further protein-protein interactions that support initiation. One of those interactions contacts eIF3, a complex factor that is itself bound to the small ribosomal subunit. This chain of interactions thus recruits the ribosomal subunit to the 5′ terminus of the RNA. The 3′ regions of an mRNA also enhance translation. One mechanism by which this can occur is through an interaction between eIF4G and poly(A) binding protein that is bound to the poly(A) tail (47). The translation enhancing contributions of the cap and poly(A) tail are synergistic (10, 46), and the most actively translated mRNAs are thought to exist in a cyclized or closed-loop state that is considered to enhance initiation and allow for improved ribosome recycling while ensuring that truncated, damaged mRNAs are poorly translated (11, 20, 40). After initial loading at the 5′ end of the RNA, the small ribosomal subunit scans in the 3′ direction along the RNA in search of an initiation site (27, 38, 39). This is usually the 5′-most AUG triplet, at which initiation frequency is in part determined by the immediate sequence context, GCC(A/G)CCAUGG being the optimal context in mammalian cells (27).

Genomes of positive-strand RNA viruses often lack a 5′ cap and/or a poly(A) tail yet must compete with cellular mRNAs

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for ribosomes to support robust viral gene expression. Viral RNAs that lack a 5′ cap often possess an internal ribosome entry site in the 5′ UTR that directs interaction with the small ribosomal subunit directly or through the binding of an initiation factor, such as eIF4G (8, 17, 39, 41). This can lead to initiation at an AUG triplet that is not 5′ proximal, as occurs with the picornaviruses (19) and the members of two genera (*Hepacivirus* and *Pestivirus*) of the family *Flaviviridae* (29). Since the genomes of DEN and other members of the genus *Flavivirus* have a 5′ cap and initiate translation at the 5′-most AUG triplet, standard cap-dependent initiation rather than internal ribosome entry has been considered to be the gene expression strategy used by these viruses (29).

The 3′ UTRs of some viral RNAs that possess a 5′ cap but lack a poly(A) tail promote translation to an extent similar to that of UTRs with a poly(A) tail, and through a similar synergistic interaction with the cap. This is true of rotavirus mRNA (49), whose 3′-terminal GACC sequence acts as a translational enhancer (5) by binding the viral protein NSP3, which competes with poly(A) binding protein for interaction with eIF4G (49). It is also true of some plant viral RNAs whose 3′ UTRs terminate in a tRNA-like structure, like those of *Tobacco mosaic virus*, *Brome mosaic virus* (12, 13), and *Turnip yellow mosaic virus* (33), although in these cases, the molecular interactions have not been identified.

We report here a systematic analysis of the properties of the 5′- and 3′-terminal regions of the DEN serotype 2 genome in directing translation in Vero cells, using a luciferase (LUC) reporter system. Both 5′ and 3′ DEN sequences were shown to participate in the regulation of expression, principally by modulating translational efficiency rather than RNA stability.

MATERIALS AND METHODS

Clones and cell culture. Dengue virus serotype 2 (DEN2) sequences were derived from the pD2/IC-30P-A infectious cDNA clone of virus strain 16681 (GenBank accession no. U87411) (23). West Nile virus sequences were derived from genomic clones of a New York flamingo isolate (GenBank accession no. AF196835). Sindbis virus sequences were obtained from pToto54, a full-length genomic clone (GenBank accession no. NC_001547) provided by James Strauss (Caltech). Vero (African green monkey kidney) cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco) containing antibiotics and 10% fetal bovine serum at 37°C in an atmosphere containing 5% CO_2.

Plasmid constructs. The basic plasmid for the in vitro transcription of reporter mRNA with DEN2 5′ and 3′ UTRs and encoding firefly LUC was constructed in the following steps to produce pDLD, shown in Fig. 1B. PCR amplification with the appropriate sequence-modifying primers was used to produce a DNA fragment comprising the DEN2 5′ UTR fused to the T7 promoter and flanked by a NotI site at the 5′ end and a PstI site at the 3′ end. The PstI site was positioned to correspond to the third and fourth codons of the reporter open reading frame (ORF) (encoding Leu-Gln). The PCR product digested with PstI was cloned into the PstI and blunted HindIII sites of pUC18. The LUC ORF was modified during PCR amplification by the addition of an in-frame PstI site in front of the second codon of the LUC ORF (Glu) and by silent mutation of the last two codons to incorporate a HindIII site immediately before the termination codon. At the same time, a BamHI site was added on the 3′ side of the LUC ORF, and the PCR product digested with PstI and BamHI was cloned into the same sites of the plasmid described above. Finally, the DEN2 3′ UTR was added into the HindIII and SmaI sites after PCR amplification to yield the 3′ UTR with a HindIII site placed immediately in front of the native DEN2 TAG stop codon and a SnaBI site appended to the 3′ end.

Derivatives of pDLD (see Fig. 1B) were made by analogous PCR amplification of the desired 5′ and 3′ UTR fragments as described above and subcloning into the NotI-PstI sites and HindIII-KpnI sites, respectively (a KpnI site is present in the polylinker downstream of SnaBI). The UTRs of rabbit α-globin mRNA (GenBank accession no. J00658) were made by annealing synthetic DNA frag-

ments. Substitution mutation and deletion variants were made by PCR-mediated mutagenesis and subcloning. A minimal 3′ UTR (Δ) was made by the removal of sequences between the BamHI and XhoI sites of an intermediate plasmid, resulting in a 15-nucleotide (nt) nonviral 3′ UTR (sequence AAAUGGAUCU CGA). The sequences of all subcloned segments were verified by DNA sequencing. Table 1 lists the relevant sequences of clones reported here.

RNA transcription and transfection of cells. Plasmids of the pDLD family were linearized by cleavage with a restriction enzyme as indicated in Fig. 1B. RNAs were produced in vitro by transcription with T7 RNA polymerase and uniformly labeled with low levels of [α-^{32}P]CTP (0.2 μCi per 20 μl reaction mixture) to facilitate quantification by liquid scintillation counting of trichloroacetic acid-precipitable material; 5′-capped RNAs were produced in the presence of a sixfold excess of m^{7}GpppG (Epicentre) over GTP (32). Template DNA was removed by digestion with RNase-free DNase, and RNA quality was analyzed via native 1% agarose gel electrophoresis.

In vitro-transcribed reporter RNA (1 pmol) was electroporated into 6×10^5 trypsinized Vero cells in electroporation buffer (25% DMEM and 75% "cytosalts," which contain 120 mM KCl, 0.15 mM $CaCl_2$, 10 mM potassium phosphate, pH 7.6, 5 mM $MgCl_2$) by using 0.2-cm cuvettes and a Gene Pulser Xcell device (Bio-Rad) as recommended by the manufacturer (the protocol can be found at the BTX website [http://www.btxonline.com/applications/protocols/mammalian/]). Electroporated cells were diluted with 4 volumes of DMEM containing 10% fetal bovine serum, transferred to tissue culture plates, and held at 37°C in an atmosphere containing 5% CO_2. Cells were harvested at various time points by lysis with 50 μl of 1 × passive lysis buffer (Promega). A number of the experiments reported in this paper were also conducted by using lipofection to deliver RNAs into cells (as used in reference 18). Although broadly similar results were obtained, we have found electroporation to be more consistent, and it permits direct estimation of translational efficiency and functional RNA half-life.

Analysis of luciferase activity. Portions of each extract (10 μl) were loaded into the wells of clear-bottom, black, 96-well plates and mixed with 50 μl of luciferase assay reagent (Promega) for luminometry in a 1450 MicroBeta TriLux counter (Wallac). Luciferase activities were normalized to the total protein concentration determined with protein assay reagent (Bio-Rad) and expressed as relative light units per mg protein.

Assessment of physical stabilities of RNAs after transfection. RNA transcripts were uniformly radiolabeled in the presence of [α-^{32}P]CTP and delivered by electroporation as usual into Vero cells. At various time points after electroporation, RNAs were extracted by use of Trizol (Invitrogen), denatured by glyoxalation, and separated by electrophoresis in 1% agarose gels run in phosphate buffer (32). After being dried, gels were exposed to phosphorimager plates, and full-length RNA bands were quantitated after phosphorimagery.

In vitro translation and assessment of the relative specific activities of LUC variants with altered N termini. LUC reporter RNAs were translated in rabbit reticulocyte lysate (Promega) in the presence of [^{35}S]methionine (Amersham). Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and dried gels were exposed to phosphorimager plates. Luciferase bands were quantitated after phosphorimagery. Dilutions of translation reactions were used for estimation of LUC activity in the standard assay. Taking into account the number of methionine residues in each form of LUC, LUC-specific activities were expressed as relative light units per pmol of LUC fusion protein.

RESULTS

Use of luciferase as a reporter for studying the effects of DEN RNA terminal regions on translational expression in primate cells. Firefly LUC has been widely used as a reporter in translational expression studies because of its sensitivity and ease of assay. We modified a cloned LUC coding region to place it under the control of a T7 promoter and to permit convenient cassette replacement of upstream and downstream control regions (Fig. 1B). In vitro transcription with T7 RNA polymerase of linearized reporter constructs produced defined mRNA transcripts that were electroporated into Vero (monkey kidney) cells for subsequent assay of LUC activity in cell lysates.

The basic LUC reporter construct with 5′- and 3′-terminal regions from DEN2 RNA, from which DLD RNA (DLD was named for *D*EN 5′ UTR/*LUC*/*D*EN 3′ UTR) can be transcribed, is shown in Fig. 1B. A unique NotI site was placed upstream of the T7 promoter, which is immediately adjacent to
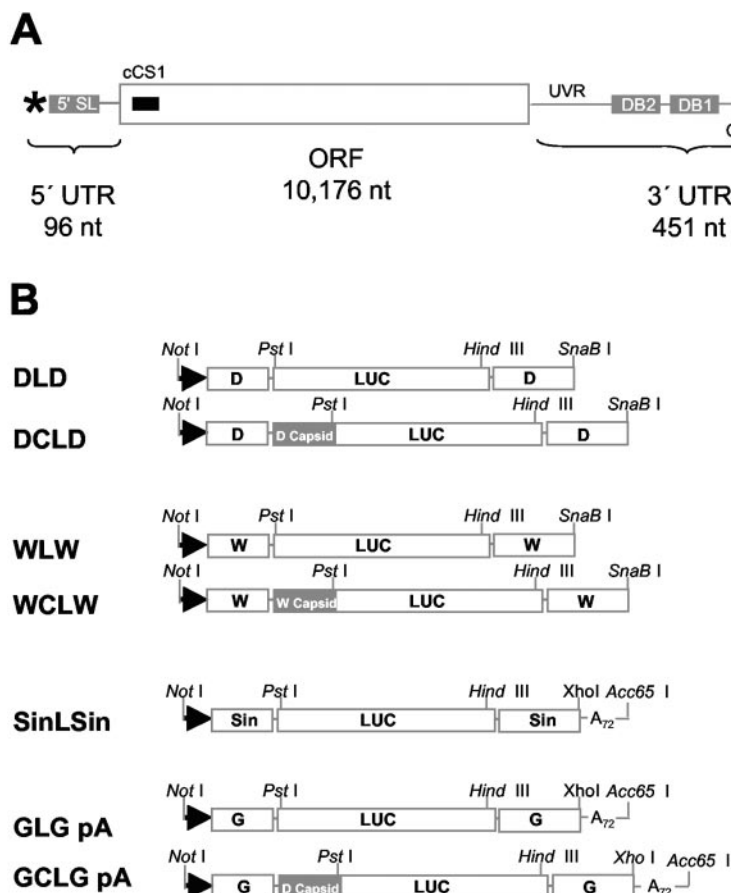
FIG. 1. Luciferase reporter constructs used for assaying the roles of flaviviral UTRs in regulating translational expression. (A) The 10.7-kb DEN2 genome is diagrammed with the 5′ cap (asterisk), 5′ UTR, the single long ORF, and the 3′ UTR that ends in a conserved stem-loop (SLA) and has no poly(A) tail highlighted. Distinctive features of the UTRs are indicated. Gray boxes indicate structural features that are conserved among mosquito-borne flaviviruses: 5′SL in the 5′ UTR (2), SLA and SLB at the 3′ end (3, 43), and the pseudo-repeated elements DB1 and DB2 further upstream in the 3′ UTR. DB1 and DB2, which resemble stalked dumbbells (36), include a conserved sequence (CS2, not shown). Black boxes indicate the conserved sequence elements, CS1 and cCS1 (16), that are complementary over 11 nucleotides. UVR represents the upstream variable region of the 3′ UTR that is not well conserved among flaviviruses. (B) LUC reporter mRNA constructs with DEN2, WNV, Sindbis virus (Sin), or rabbit α-globin UTRs. All pUC-based LUC reporter constructs have the upstream NotI site and T7 promoter (black arrowhead) and downstream KpnI, Acc65I/SacI, and EcoRI sites (not always shown). They also include an LUC coding region modified with an in-frame PstI site at the 5′ end (placed in front of the second natural codon, GAA, of the LUC ORF) and a HindIII site just upstream of the stop codon (created with silent mutations introduced into the LUC ORF). DLD RNA, which has DEN 5′ and 3′ UTRs, is made by transcription from the indicated template after linearization with SnaBI. The terminal sequences of DLD RNA differ from authentic DEN RNA by the substitution of a G for an A residue at the 5′ end and addition of UAC at the 3′ end. Translation starts with the first two authentic DEN codons and terminates with the authentic DEN UAG stop codon. DCLD RNA differs from DLD RNA by containing the first 36 codons of the DEN capsid coding region, which includes cCS1 and corresponds roughly to the capsid coding region present in flavivirus subgenomic replicons (22, 30, 35, 37). WLW and WCLW RNAs are analogous to DLD and DCLD RNAs, with all viral sequences from WNV. In these RNAs, a nonviral G residue is present at the 5′ end and an additional UAC at the 3′ end. Thirty-one codons of the capsid ORF are present in WCLW RNA. SinLSin RNA has the 59-nucleotide SIN 5′ UTR (with an additional G residue at the 5′ end to facilitate transcription), the first two codons of the SIN nonstructural polyprotein ORF, and the 318-nucleotide SIN 3′ UTR (GenBank accession no. J02362) with an XhoI site added to the 3′ end. A 72-nucleotide poly(A) tract that ends with an Acc65I site is present downstream of the XhoI site. GLG RNA contains 5′ and 3′ UTRs (36 nt and 87 nt, respectively) derived from rabbit α-globin mRNA (GenBank accession no. J00658). The first two codons of the α-globin coding region precede the two codons encoded by the PstI site of the modified LUC ORF. GCLG RNA is a derivative of GLG RNA that encodes the same capsid-LUC fusion protein as DCLD RNA. Variants with or without an A_{72} tail can be made by plasmid linearization with Acc65I or XhoI, respectively.

the 96-nt-long DEN2 5′ UTR. The 5′ UTR was fused to the first two codons of the polyprotein ORF in order to preserve the natural AUG context, and a unique PstI site was engineered in frame between these codons and the LUC ORF. To facilitate T7 transcription, the 5′ A residue of the genomic sequence was replaced with a G. At the other end of the LUC ORF, the last two codons were modified with silent mutations to incorporate a HindIII site just upstream of the stop codon. The native DEN2

stop codon and entire 451-nt-long 3′ UTR was placed between this HindIII site and a SnaBI site placed at the 3′ end (Fig. 1B). Linearization of pDLD plasmid DNA with SnaBI permits the production of transcripts with only three nonnative nucleotides at the 3′ termini. To produce RNAs with other UTRs, pDLD was modified by the replacement of NotI-PstI and HindIII-SnaBI fragments with various PCR-generated inserts.

We constructed pDCLD (DCLD was named for *DEN/*

TABLE 1. Sequences used for cloning

UTR[a]	Type of cloning	Sequence of fragment used in cloning[b]
T7-5′ DEN	NotI-PstI	<u>CGGCCG</u>CTAATACGACTCACTATAGGTTGTTAGTCTACGTGGACCGACAAAGACAGATTCTTTGAGGGAGCTAAGCTCAACGTAGTTCTAACAGTTTTTTAATTAGAGAGCAGATCTCT**GATGA**AT<u>CTGCAG</u>
T7-5′ DEN/DEN capsid	NotI-PstI	<u>CGGCCG</u>CTAATACGACTCACTATAGGTTGTTAGTCTACGTGGACCGACAAAGACAGATTCTTTGAGGGAGCTAAGCTCAACGTAGTTCTAACAGTTTTTTAATTAGAGAGCAGATCTCT**GATG**AATAACCAACGGAAAAAGGCGAAAAACACGCCTTTCAATATGCTGAAACGCGAGAGAAACCGCGTGTCGACTGTGCAACAGCTGACAAAGAGATTCTCACTTGGA<u>CTGCAG</u>
T7-5′ globin	NotI-PstI	<u>CGGCCG</u>CTAATACGACTCACTATAGACACTTCTGGTCCAGTCCGACTGAGAAGGAACCAC**CATG**GT<u>GCTGCAG</u>
T7-5′ globin/DEN capsid	NotI-PstI	<u>CGGCCG</u>CTAATACGACTCACTATAGACACTTCTGGTCCAGTCCGACTGAGAAGGAACCAC**CATG**AATAACCAACGGAAAAAGGCGAAAAACACGCCTTTCAATATGCTGAAACGCGAGAGAAACCGCGTGTCGACTGTGCAACAGCTGACAAAGAGATTCTCACTTGGA<u>CTGCAG</u>
T7-5′ WNV	NotI-PstI	<u>CGGCCG</u>CTAATACGACTCACTATAGAGTAGTTCGCCTGTGTGAGCTGACAAACTTAGTAGTGTTTGTGAGGATTAACAACAATTAACACAGTGCGAGCTGTTTCTTAGCACGAAGATCTCG**ATG**TCT<u>CTGCAG</u>
T7-5′ WNV/WNV capsid	NotI-PstI	<u>CGGCCG</u>CTAATACGACTCACTATAGAGTAGTTCGCCTGTGTGAGCTGACAAACTTAGTAGTGTTTGTGAGGATTAACAACAATTAACACAGTGCGAGCTGTTTCTTAGCACGAAGATCTCG**ATG**TCTAAGAAACCAGGAGGGCCCGGCAAGAGCCGGGCTGTCAATATGCTAAAACGCGGAATGCCCGCGTGTTGTCCTTGATTGGACTGAAG<u>CTGCAG</u>
T7-5′ Sin	NotI-PstI	<u>CGGCCG</u>CTAATACGACTCACTATAGATTGACGGCGTAGTACACACTATTGAATCAAACAGCCGACCAATTGCACTACCATCACA**ATG**GAG<u>CTGCAG</u>
3′ DEN	HindIII-SnaBI	<u>AAGCTT</u>**TAG**AAAGCAAAACTAACATGAAACAAGGCTAGAAGTCAGGTCGGATTAAGCCATAGTACGGAAAAAACTATGCTACCTGTGAGCCCCGTCCAAGGACGTTAAAAGAAGTCAGGCCATCATAAATGCCATAGCTTGA————————AATGGAATGGTGCTGTTGAATCAACAGGTTCT<u>TACGTA</u>
3′ globin	HindIII-XhoI	<u>AAGCTT</u>**TAA**GCTGGAGCCTGGGAGCCGGCCTGCCCTCCGCCCCCCCCCATCCCCGCAGCCCACCCCTGGTCTTTGAATAAAGTCTGAGTGAGTGGCA<u>CTCGAG</u>
3′ WNV	HindIII-SnaBI	<u>AAGCTT</u>**TAG**ATATTTAATCAATTGTAAATAGACAATATAAGTATGCATAAAAGTGTAGTTTTATAGTAGTATTTAGTGGTGTTAGTGTAAATAGTTAAGAAAATTTTGAGGAGAAAGTCAGGCCGGGAAGTTCCCGCCACCGGAAGT————————ATGGTGGCTGGTGGTGCGAGAACACAGGATC<u>TACGTA</u>
3′ Sin	HindIII-XhoI	<u>AAGCTT</u>**TGA**CCGCTACGCCCCAATGATCCGACCAGCAAAACTCGATGTACTTCCGAGGAACTGATGTGCATAATGCATCAGGCTGGTACATTAGATCCCCGCTTACCGCGGGCAATATAGCAACACTAAAAACTCGATGTACT————————TTTATTAATCAACAAAATTTTGTTTTTAACATTT<u>CTCGAG</u>
3′ Δ	HindIII-XhoI	<u>AAGCTT</u>**TAA**AATGGAT<u>CTCGAG</u>

[a] Sin, Sindbis virus; Δ, 15-nt nonviral PCR product.
[b] Restriction enzyme sites used for cloning are underlined. Initiation codons and stop codons are in bold. Connecting lines represent sequences not shown in the table.

*C*apsid/*L*UC/*DE*N) (Fig. 1B) to investigate the influence of the 5′-proximal part of the DEN ORF, which includes the cCS1 element that permits RNA cyclization by hybridization with the CS1 element in the 3′ UTR (Fig. 1A). pDCLD includes the first 36 codons of the DEN capsid coding region fused in front of the PstI site and placed in frame upstream of the LUC coding sequence (Fig. 1B). This results in the production of a capsid-LUC fusion protein. To compare translation driven by viral sequences to that driven by UTRs from a highly expressed cellular mRNA, we have made pGLGpA and pGCLGpA (Fig. 1B). These constructs include the 5′ and 3′ UTRs derived from rabbit α-globin mRNA and a poly(A) tail 72 residues long. In pGLGpA, the first two codons of the α-globin ORF are fused to the PstI site and in frame with the LUC coding sequence, while in pGCLGpA, the entire coding region is identical to that in pDCLD. RNAs with and without a poly(A) tail can be made from pGLGpA and pGCLGpA after linearization with Acc65I and XhoI, respectively.

The specific activities of all the forms of N-terminally modified LUC made by these and other RNAs studied in this paper have been determined and have been verified to be similar (Fig. 2). This was assessed by determination of the light yields from known amounts of [^{35}S]methionine-labeled LUC pro-teins synthesized by in vitro translation. We have previously observed consistent specific activities for LUC forms with different N-terminal extensions (32).

Flavivirus UTRs support highly efficient translation in Vero cells. To assess the overall efficiency with which flavivirus UTRs are able to support translation in Vero cells, we studied LUC expression from reporter RNAs with DEN or WNV UTRs: DLD, DCLD, WLW (named for *W*NV 5′ UTR/*L*UC/*W*NV 3′ UTR), and WCLW (named for *W*NV/*C*apsid/*L*UC/*W*NV) RNAs (Fig. 1B). Expression was compared to that from GLGpA RNA and from SinLSin RNA, which have α-globin and Sindbis virus UTRs, respectively (Fig. 1B). SinLSin RNA was included as a representative of a virus capable of rapid amplification and highly efficient gene expression (44). All RNAs contained 5′ m^{7}GpppG caps (Fig. 3). In time courses following LUC expression up to 9 h after RNA delivery by electroporation, DLD, WLW, and SinLSin RNAs supported the synthesis of considerably more LUC activity than did GLGpA RNA (Fig. 3A).

We derive three parameters relevant to UTR function from LUC expression time courses. Estimates of the maximum rate of increase in LUC activity (linear rate [see Fig. 3 to 7]), which is observed during an early phase of linear increase, are taken
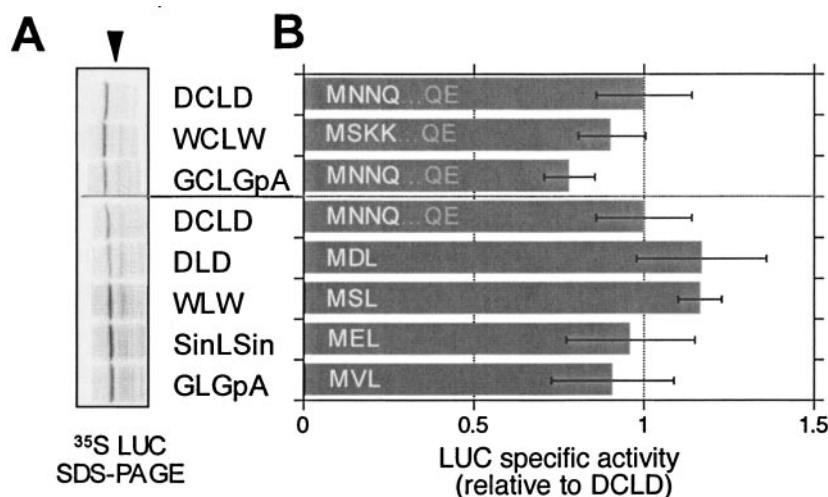
FIG. 2. LUC variants with N-terminal fusions have similar specific activities. The indicated RNAs were translated in a rabbit reticulocyte lysate in the presence of [^{35}S]methionine. (A) Translation products were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10%); the major product, LUC, is indicated by an arrowhead. These samples are shown to indicate the quality of the LUC product, not the translational efficiencies of the various RNAs. (B) Relative specific activities (relative light units per pmol) of LUC proteins made in reticulocyte lysates (averages from three replicates with each error bar representing one standard deviation). The N-terminal sequences of LUC proteins made by these RNAs are indicated (single-letter amino acid code); intervening capsid sequences are indicated by three periods.

to reflect translational efficiencies (9, 32). The maximum accumulation of LUC activity reflects the expression capacity of each RNA. This capacity is influenced by the translational efficiency of the mRNA and by the stabilities of the mRNA and translated protein. The combined RNA and protein stabilities can be monitored by determining the longevity of continued LUC expression. From the time courses, we estimate the half-life of LUC expression as the time taken for the maximum rate of expression (discussed above) to fall to half that rate. Rates are determined as the slopes of the lines of best fit for the expression time courses. For reporter RNAs encoding the same protein, differences in LUC half-life reflect differences in RNA functional stability (the RNA's capacity for directing protein synthesis) (9, 32). In cases in which the N termini of the encoded LUC proteins are different, differences in protein stability may also be reflected in the LUC protein half-life. The expression half-lives of LUC expressed from DLD, WLW, SinLSin, and GLGpA RNAs were all similar (Fig. 3A). LUC synthesis from the RNAs with viral UTRs were expressed with two- to threefold higher translational efficiency than LUC expressed from GLGpA RNA (Fig. 3A, linear rate). The flaviviral reporter RNAs expressed LUC at least as efficiently as did SinLSin RNA. LUC expression from WLW and SinLSin RNAs consistently appeared earlier than from DLD RNA (Fig. 3A), suggesting differences in the ability to recruit ribosomes for the initial round of translation.

Variants of DLD and WLW that included the first 36 and 31 codons, respectively, of each viral capsid coding region (DCLD and WCLW RNAs [Fig. 1B]) were constructed to more closely represent the translation of natural viral RNAs. The presence of additional 5′ viral sequences allows local folding into a predicted stem-loop that includes the initiation AUG (mfold [52]) and long-distance pairing between CS1 and cCS1. Further, under some circumstances, the proximal part of an ORF can affect the efficiency of its own translation (7, 26). The same

fragment of the DEN capsid ORF was present in GCLGpA RNA as a control. The specific activities of the capsid-LUC fusion proteins varied little from those of the corresponding LUC proteins lacking the capsid ORF (Fig. 2B), while expression half-lives were marginally shorter (Fig. 3). The decrease in LUC activities after 6 h suggests a shorter half-life for the capsid-LUC fusion proteins.

The efficiency and maximal expression of LUC from DCLD and WCLW RNAs were two- to fivefold lower than those of LUC from DLD and WLW RNAs, respectively (Fig. 3B and A, respectively). Nevertheless, DCLD and WCLW RNAs supported strong LUC expression at levels similar to that of GLGpA RNA. The presence of DEN capsid coding sequences in GCLGpA RNA had little effect on LUC expression (Fig. 3, compare GCLGpA and GLGpA), in contrast to their effect on DLD RNA. Decreased expression is, thus, not a property of the encoded protein but likely a property of the RNA (such as different folding). The capsid-coding regions include cCS1, thereby permitting long-distance hybridization to the complementary sequence, CS1, that is present in the viral, but not globin, 3′ UTRs. Together with additional long-distance 5′-3′ pairing involving sequences just upstream of the initiation AUG as suggested recently (48), these RNA-RNA interactions may dampen the efficiency of ribosome access in the case of RNAs with viral 5′ and 3′ terminal regions.

Translational regulation by DEN 5′ and 3′ UTRs. Efficient translation of cellular mRNAs depends heavily on the 5′ cap and 3′ poly(A) tail, which enhance expression synergistically (10, 46). This response was confirmed for Vero cells by using transcripts from pGCLGpA. Detecting synergy requires study of the expression of capped and uncapped versions of an RNA, as well as variants with and without a poly(A) tail. In the presence of a 5′ cap, the addition of a polyadenylated globin 3′ UTR (3′-GpA) enhanced translational efficiency (linear rate) by 108-fold with reference to an RNA with a minimal vector-
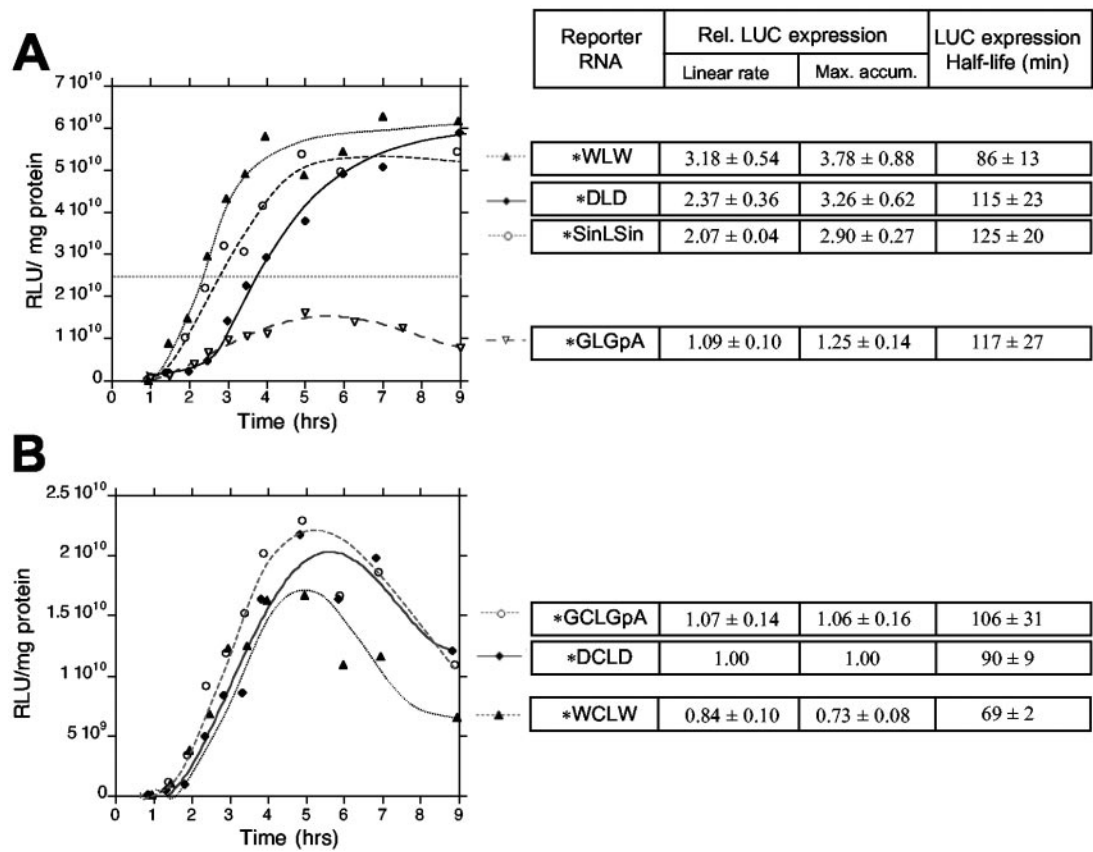
FIG. 3. Efficient LUC expression from 5′-capped flavivirus reporter mRNAs in Vero cells. Vero cells were electroporated with the indicated RNAs, and LUC extracts were prepared after various incubation times. LUC activities and protein levels in each extract were assayed to determine corrected LUC yields (relative light units [RLU] per mg protein). Representative time courses from single experiments (average of duplicates) are shown in the time courses of panels A and B. The tabulated results report LUC expression relative to that from DCLD RNA, with the (maximal) linear rate representing translational efficiency. The maximum LUC accumulation for each RNA is also reported, as is the LUC expression half-life, which reflects the combined RNA and LUC protein stability. The tabulated results reflect averages from at least two separate transfection experiments performed on different days, involving duplicate sets of independently made RNA transcripts in each case. Standard errors are indicated. The asterisk in front of each RNA name represents a 5′ m⁷GpppG cap.

derived 3′ UTR (Fig. 4A and C; compare *GCLGpA and *GCLΔ). Far less enhancement (5.5-fold) in response to adding 3′-GpA when RNAs were uncapped was observed (Fig. 4B and C; compare GCLGpA and GCLΔ). The ratio between these responses (108/5.52 = 19.6) represents synergistic enhancing effects between the 5′ cap and polyadenylated 3′ UTR, implying collaboration between these features. Note that the presence of 3′-GpA also increased the LUC expression half-life by threefold (82/27) (Fig. 4A). Since GCLGpA and GCLΔ RNAs encode the same form of LUC, we can conclude that there is a threefold difference in the functional half-lives of these RNAs.

Similar analyses were conducted to study the contribution of the DEN 3′ UTR to translational expression. Like the 3′-GpA, the DEN 3′ UTR is clearly important for high-level expression of LUC (Fig. 4A, compare *DCLD and *DCLΔ RNAs). The addition of the DEN 3′ UTR to DCLΔ RNA (to produce DCLD RNA) enhanced translational efficiency 32-fold in the presence of a 5′ cap (Fig. 4A and C; compare *DCLD and *DCLΔ) and 10.5-fold in the absence of a cap (Fig. 4B and C; compare DCLD and DCLΔ). This indicates the existence of a synergistic interaction between the cap and DEN 3′ UTR

(synergy value, 32.3/10.5 [3.1]), although the interaction is considerably less strong than that between a cap and 3′-GpA.

Studies with the uncapped forms of RNAs, which support relatively low levels of LUC expression, emphasized the importance of a 5′ cap. Among uncapped RNAs, however, elevated levels of LUC expression from RNAs with DEN UTRs were observed. This is obvious in the case of DCLD RNA in the time course shown in Fig. 4B. An enhancing effect of 5′ DEN sequences can be seen for DCLΔ RNA in comparison with GCLΔ RNA, while an effect of the DEN 3′ UTR is evident when comparing expression levels from GCLD and GCLGpA RNAs (Fig. 4B and C). The enhancing effect was stronger for the DEN 5′ UTR, which supported the synthesis of LUC 6 to 12 times more efficiently (Fig. 4B and C, linear rates) than 5′ G in RNA counterparts with the same 3′ UTR. No such 5′ DEN-dependent enhancement in the capped state was seen, since capped DCLD, GCLD, and GCLGpA RNAs were similarly expressed (Fig. 4A).

LUC expression half-life estimates indicated that the GpA and DEN 3′ UTRs and the 5′ cap all contribute to RNA stability. Capped DCLD, GCLD, and GCLGpA RNAs (which encode the same form of LUC) had similar functional half-
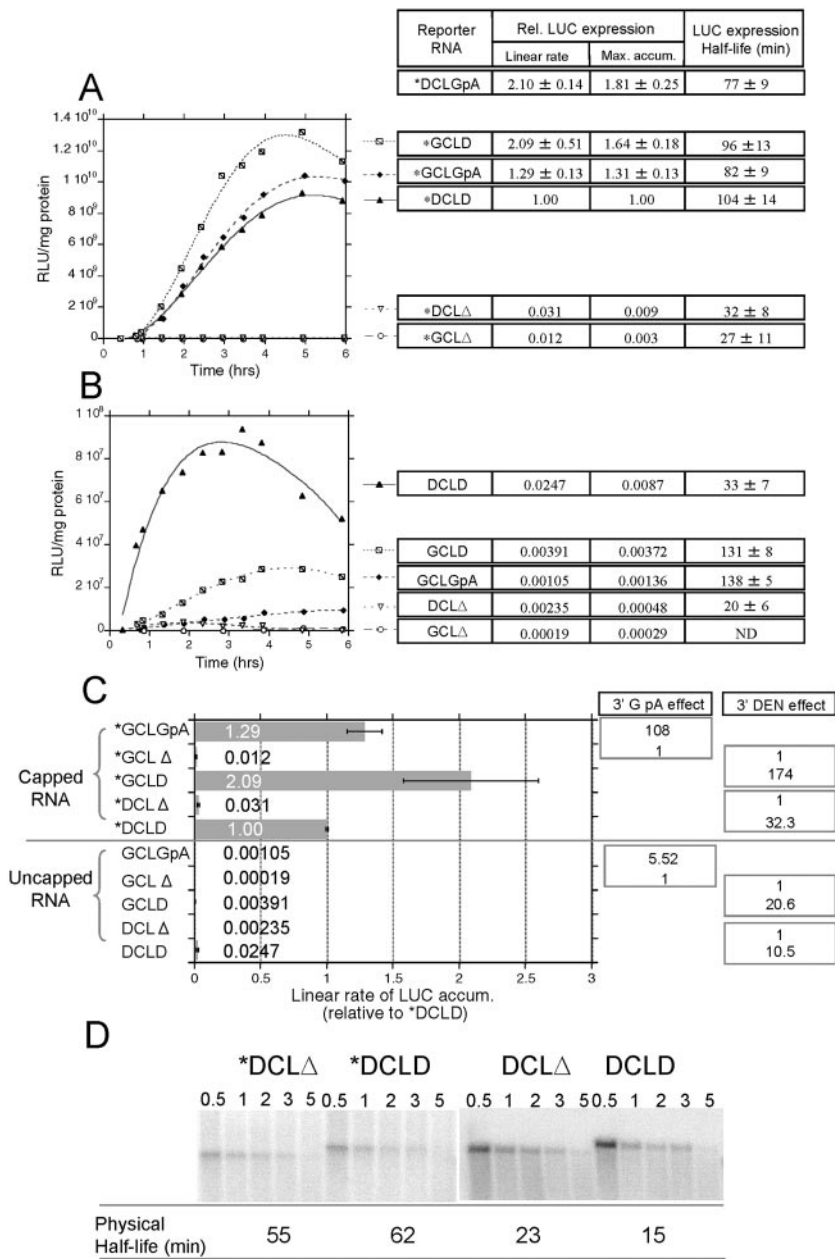
Reporter RNA	Rel. LUC expression		LUC expression Half-life (min)
	Linear rate	Max. accum.	
*DCLGpA	2.10 ± 0.14	1.81 ± 0.25	77 ± 9
*GCLD	2.09 ± 0.51	1.64 ± 0.18	96 ±13
*GCLGpA	1.29 ± 0.13	1.31 ± 0.13	82 ± 9
*DCLD	1.00	1.00	104 ± 14
*DCLΔ	0.031	0.009	32 ± 8
*GCLΔ	0.012	0.003	27 ± 11

DCLD	0.0247	0.0087	33 ± 7
GCLD	0.00391	0.00372	131 ± 8
GCLGpA	0.00105	0.00136	138 ± 5
DCLΔ	0.00235	0.00048	20 ± 6
GCLΔ	0.00019	0.00029	ND

FIG. 4. Contributions of DEN 5′ and 3′ UTRs to translational expression in Vero cells. Data of LUC expression from the indicated capped (A) or uncapped (B) RNAs are as described in the legend to Fig. 3. DCLΔ and GCLΔ RNAs have 3′ UTRs comprised of only 15 vector-derived nucleotides (UAAAAUGGAUCUCGA). Note the different scales on the *y* axes in panels A and B. Expression data for capped (*) DCLGpA RNA are tabulated at the top of panel A, but the graphed time course is not shown, in order to avoid cluttering the graph. For clarity, standard errors of LUC expression have been omitted for the less-efficiently expressed RNAs in panels A and B; standard errors for these determinations averaged about 20% of the means. RLU, relative light units. ND, not determined. (C) The relative linear rates of LUC expression from the various RNAs are compared graphically. (D) Assay of the physical stabilities of selected ^{32}P-labeled transcripts electroporated and incubated in Vero cells for the indicated times before extraction and analysis in the denatured state in a 1% agarose gel. The estimated half-lives are written at the bottom.

lives, and the removal of the 3′ UTR decreased the half-life by about threefold (Fig. 4A, compare *DCLΔ and *GCLΔ RNAs). Uncapped DCLD RNA had a half-life similar to that of capped DCLΔ or GCLΔ RNAs, evident from the early peak in LUC activity (see the DCLD time course in Fig. 4B). While RNA stability differences associated with the 5′ cap and 3′ UTR are important, they were much smaller in magnitude than the enhancements in translational efficiency provided by

these features. Analyses of the physical stabilities of RNAs during expression time courses confirmed that RNA stability differences were relatively small among the RNAs studied in Fig. 4 (Fig. 4D). In these assays, ^{32}P-labeled RNAs were electroporated and incubated in cells before being extracted and examined by electrophoresis (see Materials and Methods).

Expression from DCLGpA and GCLD RNAs was studied to assess whether there are any cooperative effects between DEN

5′ and 3′ UTRs. LUC expression from both of these capped RNAs was comparable to expression from capped DCLD and GCLGpA RNAs (Fig. 4A), clearly indicating that the DEN UTRs do not need to be coupled to support strong translation. The addition of the DEN 3′ UTR to GCLΔ RNA enhanced translational efficiency 174-fold in the presence of a 5′ cap and 20.6-fold with no cap (Fig. 4C). Thus, the synergy between the 5′ cap and DEN 3′ UTR (synergy value, 174/20.6 [8.4]) for these RNAs with a globin 5′ UTR is stronger than that observed for RNAs with the DEN 5′ UTR (synergy value of 3.1; see above). As evident when comparing DCLD and DCLΔ RNAs, the DEN 3′ UTR in GCLD RNA enhanced the RNA functional half-life about threefold (Fig. 4A). We conclude that the DEN 3′ UTR is a general enhancer of translation and RNA stability whose action does not require a matched DEN 5′ UTR.

Complementarity between cyclization sequences CS1 and cCS1 is not needed for translation. The analyses already discussed failed to detect any codependency between 5′ and 3′ DEN sequences in regard to the control of translation. To explore this issue further, we wished to test directly for a translational role of the conserved cyclization sequences and their potential long-distance hybridization. The RNAs of Fig. 5A represent a set in which CS1 and cCS1 have replaced one another singly and together, resulting in DCmLD and DCLDm RNAs that have diminished potential for long-distance cCS1/CS1 interaction and double-mutant DCmLDm RNA, in which CS1 and cCS1 have been switched and cyclization is possible. Note that one nucleotide of each transposed conserved sequence was changed to avoid introducing a stop codon with CS1 placed in the 5′ location. Expression from capped forms of these RNAs in Vero cells was studied.

Replacement of cCS1 with the modified CS1 sequence had little impact on LUC expression or expression half-life (Fig. 5, compare *DCLD and *DCmLD). By contrast, replacement of CS1 with the modified cCS1 decreased LUC expression efficiency to 19%, with only a small effect on expression half-life (Fig. 5, compare *DCLD and *DCLDm). CS1 and cCS1 thus appear to have differential roles in translational regulation. Significantly, the inability of the double mutation (*DCmLDm) to rescue the depressed LUC expression level of *DCLDm RNA (Fig. 5B and C) suggests that RNA cyclization via cCS1/CS1 interaction is not required for the translation of the reporter RNAs used here. The minimal differences in the half-lives of LUC expression from the RNAs in Fig. 5 indicate that these RNAs have similar stabilities.

Contribution of conserved elements in the 3′ UTR to translational expression. The results described above indicated an involvement of CS1 in translation. In order to identify contributions from other elements in the 3′ UTR, the conserved features SLA, SLB, DB1, and DB2, as well the upstream variable region (UVR) (Fig. 1A and 6A), were deleted in turn from the 3′ UTR of capped DCLD RNA. The endpoints of deletions were chosen to cleanly delete the predicted structures as shown in Fig. 6A, and this was confirmed by mfold (52) predictions of the folding of the 3′ UTRs. A large 3′ truncation was made by transcription from a pDCLD template linearized with NcoI, which cleaves at DEN nucleotide 10470 (Fig. 6A). This produced a variant of DCLD RNA (Fig. 6, */NcoI) truncated near the upstream end of DB2 and thus lacking SLA,

SLB, CS1, DB1, and most of DB2. LUC expression from capped RNAs in Vero cells was studied (Fig. 6).

The deletion of either of the two 3′-terminal stem-loops SLA and SLB decreased the efficiency of LUC translation to about half (Fig. 6B, linear rate). The deletion of DB1 resulted in a smaller, though significant, decrease in the efficiency of LUC translation, while the deletion of DB2 had little effect (Fig. 6C). The simultaneous deletions of DB1 and DB2 reduced the efficiency of translation to 25%, suggesting redundancy in the contributions of these quite similar features. The deletion of the UVR, just downstream of the stop codon, also resulted in a relative translational efficiency of 25% (Fig. 6D).

Translational enhancement provided by the 3′ UTR thus seems to derive from several features, including contributions by each of the conserved features. The individual deletions of these features led to a partial loss of activity. Expression from each of the RNAs with a single element deleted was higher than that from RNA entirely lacking a DEN 3′ UTR (Fig. 7A, *DCLΔ). Presence of the UVR feature alone (Fig. 7A, */NcoI RNA) increased expression only slightly from this basal level (Fig. 7A), emphasizing the importance of combined contributions to translational enhancement by the 3′ UTR.

Interestingly, DCLG RNA, which has the 87-nucleotide-long globin 3′ UTR but no poly(A) tail, supported the same basal level of LUC expression as DCLΔ RNA (Fig. 7). High levels of expression were dependent on the poly(A) tail (Fig. 7A, *DCLGpA RNA). This emphasizes the similar roles of the DEN 3′ UTR and poly(A) tail in enhancing translation.

It is important to note that no noteworthy differences in functional RNA stability were observed for the RNAs with various partial deletions of the DEN 3′ UTR, although most of these capped RNAs had somewhat shorter half-lives than capped DCLD RNA (Fig. 7B); this conclusion was supported by an analysis of the physical stabilities of these RNAs (not shown). The RNAs containing partial 3′ deletions were all more stable than DCLΔ RNA (Fig. 7B). It appears that, as for translational enhancement, RNA stabilization (roughly threefold) provided by the DEN 3′ UTR (noted earlier) derives from multiple features in the UTR. The globin 3′ UTR also appears to enhance RNA stability (twofold stabilization observed for DCLG RNA relative to DCLΔ [Fig. 7B]), with little if any further stabilization provided by a poly(A) tail (Fig. 7B, DCLGpA RNA). The contributions to translational enhancement were just the opposite, being primarily derived from the poly(A) tail and not measurable from the 3′ G sequence (Fig. 7A).

Although it has been observed that a 3′ UTR can stabilize RNA and enhance translation by virtue of its length, in a sequence-nonspecific effect (45), this is unlikely to be a consideration among the 3′ UTR domain deletions. Those deletions remove no more than 154 nt (ΔDB1 + 2), leaving at least a 297-nt-long UTR, and, conversely, the addition of the 87-nt 3′ G did not enhance translation (Fig. 7A). The nonspecific length effects were observed for 3′ UTRs shorter than 27 nt (45).

DISCUSSION

Flaviviral 5′- and 3′-terminal genome sequences support highly efficient translational expression. Using a luciferase reporter system and comparison with reporter RNAs that support highly efficient translation, we have shown that the 5′- and
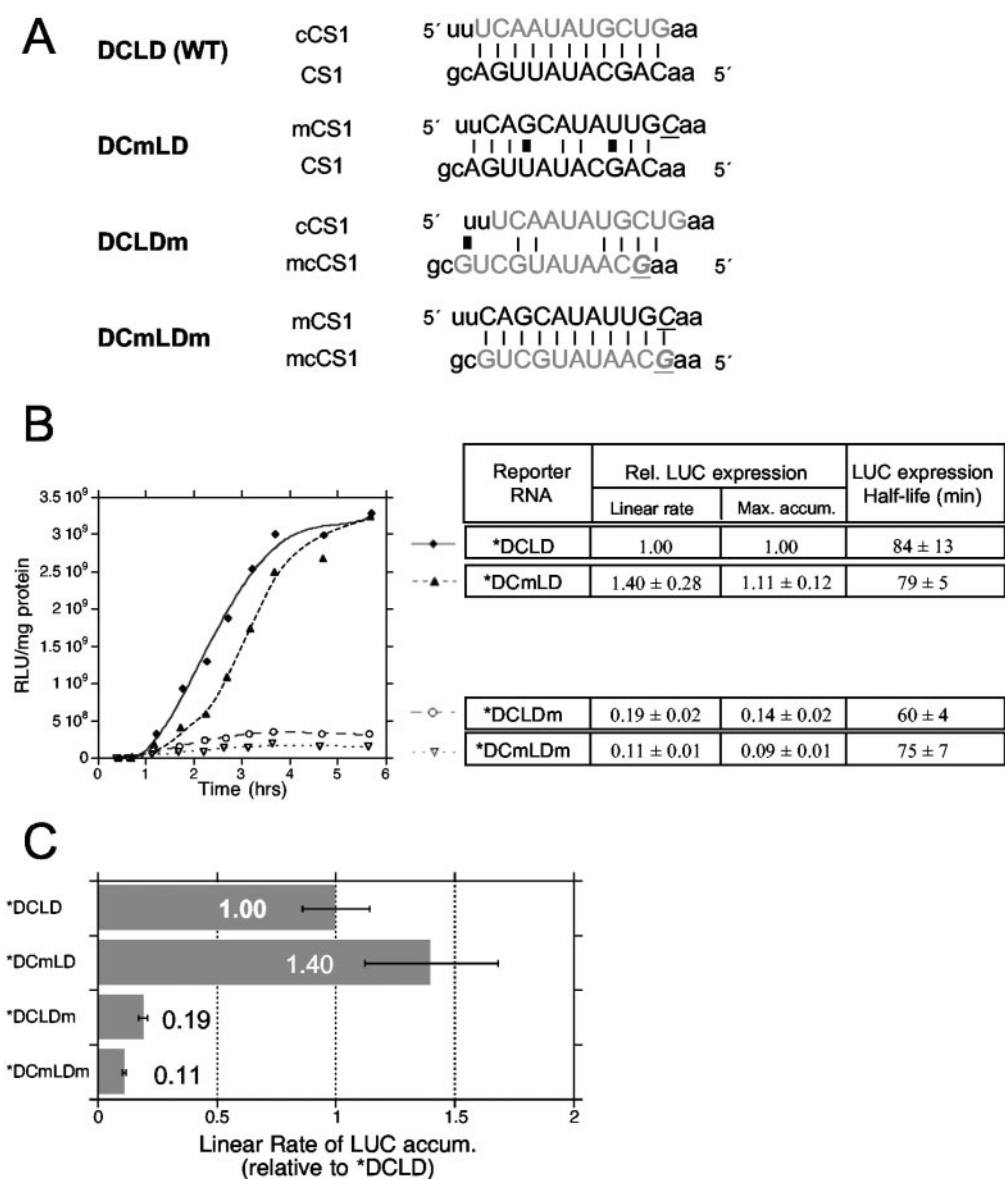
FIG. 5. Complementarity between cCS1 and CS1 is not required for LUC expression from DCLD RNA. (A) The sequences of wild-type and variant cyclization sequences cCS1 (5′, indicated in gray lettering) and CS1 (3′, black lettering) are shown. The wild-type cCS1 and CS1 sequences, which form a perfect 11-bp duplex, are shown in uppercase letters. The three mutant RNAs were constructed by transposing these sequences to the other end of the genome. Single-base changes in mCS1 and mcCS1, indicated by underlined italics, were necessary to avoid the insertion of a UGA stop codon within the capsid ORF when CS1 was moved to the 5′ location. The most stable long-distance base-pairing schemes are shown, with the sequence in the 5′ region of the mRNA in the upper line. (B) Data of LUC expression from the indicated capped (*) RNAs are as described in the legend to Fig. 3. (C) The relative linear rates of LUC expression from the various RNAs are compared graphically. RLU, relative light units.

3′-terminal regions from the DEN2 and WNV genomes are able to support strong translation in Vero cells. Translational expression levels from 5′-capped DCLD and WCLW RNAs with 5′ and 3′ sequences from DEN and WNV, respectively, were similar to the level of expression from capped GLGpA RNA and only two to three times lower than the level of expression from SinLSin RNA (Fig. 3). GLGpA RNA has UTRs from the α-globin mRNA, while SinLSin RNA has UTRs derived from Sindbis virus, an alphavirus that has been developed as a gene expression vector because of its ability to support potent translational expression (1).

The viral sequences present in DCLD and WCLW RNAs included part of the capsid coding region in addition to the 5′ and 3′ UTRs. The capsid coding regions were included because they contain the conserved cCS1 sequence element that permits potential cyclization of the RNA by annealing to its complement, CS1, present in the 3′ UTR. A surprising finding was that the omission of the capsid coding regions (DLD and WLW RNAs) increased translational expression by two- to fourfold (Fig. 3). This is not due to an elongational block of translation associated with the capsid ORF, since LUC was similarly expressed from GLGpA and GCLGpA RNAs (Fig.
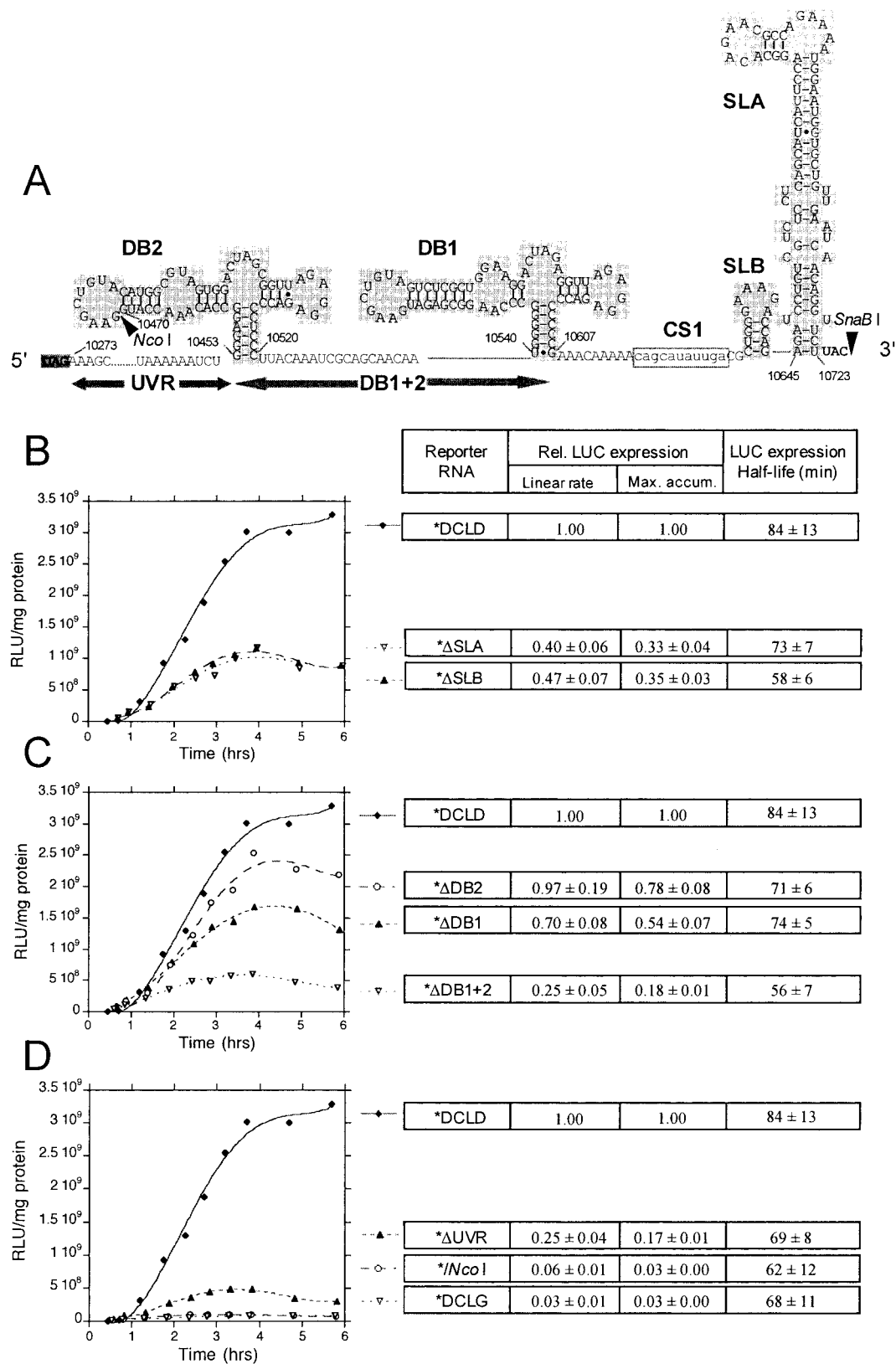
FIG. 6. Several features from the DEN 3′ UTR contribute to efficient translation. (A) The sequences of conserved features of the DEN2 3′ UTR are shown in their proposed foldings. The native DEN stop codon UAG is shown on the left, and the additional nonviral nucleotides at the 3′ end of DCLD RNA are shown on the right. The NcoI restriction site used for producing the */NcoI variant of DCLD RNA is marked. The foldings of DB1 and DB2 are according to reference 37, while the foldings of SLA and SLB are according to references 3 and 48. The numbered nucleotides (numbering from the 5′ end of the DEN genome) represent the deletion boundaries in the deletion derivatives of DCLD studied in panels B through D. (B to D). Data of LUC expression from the indicated capped (*) RNAs is as described in the legend to Fig. 3. The y axes are the same in each case. RLU, relative light units.
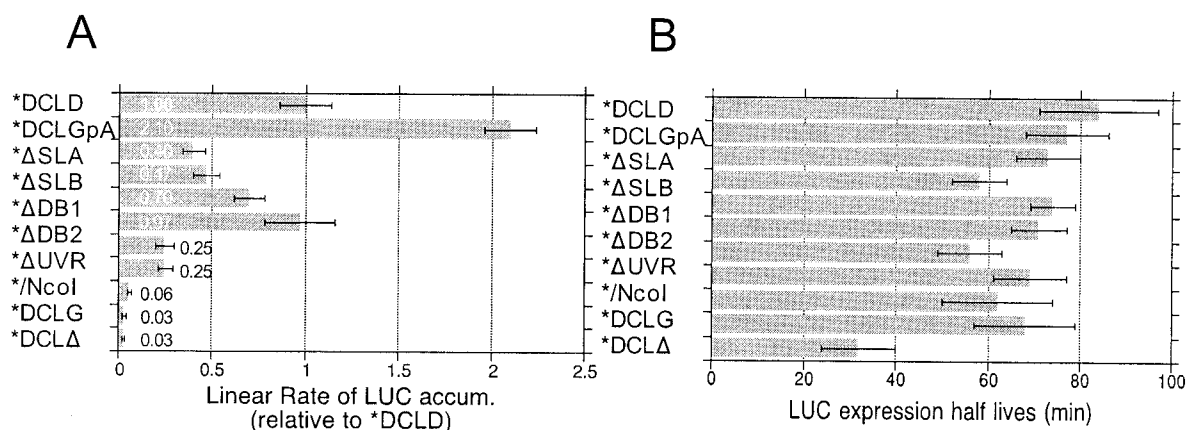
FIG. 7. Translational efficiencies and RNA functional half-lives of 5′-capped DCLD variants with deletions in the 3′ UTR. Graphical representations of relative translation efficiencies (linear rates of LUC accumulation) (A) and LUC expression half-lives (B) are shown for the indicated RNAs. Because all of the RNAs encode the same form of LUC, LUC expression half-lives are proportional to RNA functional half-lives. Data are taken from Fig. 4 and 6. Error bars represent standard errors.

3). We have also verified that the LUC and capsid-LUC fusion proteins have similar light-emitting specific activities (Fig. 2). It seems most likely that the presence of the proximal part of the capsid ORF lessens translational efficiency by affecting ribosome initiation, perhaps by altering the folding of the 5′ UTR through local interactions or as a consequence of long-distance hybridization with CS1.

In any case, it is clear that the capped flaviviral 5′ UTRs are capable of directing very high translational expression in Vero cells. This was rather unexpected in view of the conserved secondary structure positioned immediately adjacent to the 5′ end (2) and suboptimal consensus around the initiation AUG (UCUCUGAUGA for DEN and AUCUCGAUGU for WNV). Secondary structure at the 5′ terminus is detrimental to cap-dependent translation initiation (25), and weak consensus nucleotides at the −3 and +4 positions (italicized respectively in the sequences above) can diminish translation by up to 10-fold (−3A or −3G and +4G is optimal [27]). Schrader and Westaway (42) have also commented on the efficient translation of flaviviral (Kunjin virus) RNA in Vero cells despite suboptimal AUG context.

The DEN 3′ UTR enhances translation and RNA stability in a way similar to that of a poly(A) tail. The contribution of the DEN 3′ UTR to translational expression was studied by determining the effect of replacing a short nonviral 3′ UTR (DCLΔ or GCLΔ RNAs) with the DEN 3′ UTR (DCLD and GCLD RNAs). For capped RNAs with either the DEN or globin 5′ UTRs, the DEN 3′ UTR significantly increased RNA stability (about a threefold increase in functional half-life) (Fig. 4A). Far greater increases in translational efficiency were observed, represented by the maximum LUC expression rate in our assays (Fig. 3 to 6, linear rates). In the presence of a DEN 5′ UTR, the rate was increased 32.3-fold (Fig. 4C, compare *DCLD and *DCLΔ), while in the presence of a globin 5′ UTR, the increase was 174-fold (Fig. 4C, compare *GCLD and *GCLΔ). These responses are very comparable to those provided by a polyadenylated 3′ UTR. Capped GCLGpA RNA was three times as stable and was translated with 108 times the efficiency of capped GCLΔ RNA (Fig. 4C), and capped

DCLGpA RNA was translated with 70 times the efficiency of capped DCLΔ RNA (Fig. 7A). The ability of the DEN 3′ UTR to enhance translational efficiency and RNA stability in the presence of either DEN or globin 5′ sequences demonstrates this to be a free-standing regulator that is not codependent on viral 5′ sequences. Stimulation of the translation of LUC reporter mRNAs by the DEN2 3′ UTR has also recently been reported by Holden and Harris (18). Extending those studies, our kinetic analyses have enabled estimation of the contributions of both increased RNA stability and translational efficiency to expression enhancement by the 3′ UTR.

The poly(A) tail is known to enhance translation synergistically with a 5′ cap (10, 46). Such synergy is established by determining the extent to which the addition of a poly(A) tail has a greater effect in the presence of a cap than in the absence of a cap. The 3′-GpA enhanced translational efficiency 19.6 (108/5.52) times more when a cap was present (Fig. 4C, GCLGpA and GCLΔ RNAs), confirming the strong synergy that others have reported. Similar analyses of the role of the DEN 3′ UTR also indicated the existence of synergy with a 5′ cap, although this synergy was weaker than that observed for the case with a poly(A) tail and it differed for different 5′ UTRs. Thus, the cap/3′ DEN synergy value was 8.4 for RNAs with a globin 5′ UTR (ratio for GCLD and GCLΔ RNAs, 174/20.6 [Fig. 4C]) and 3.1 for RNAs with a DEN 5′ UTR (ratio for DCLD and DCLΔ RNAs, 32.3/10.5 [Fig. 4C]).

If cap/poly(A) synergy reflects the existence of bridging interactions that cyclize the mRNA as described in the introduction, we may think of the DEN 3′ UTR recruiting proteins capable of interacting with translation initiation factors assembled around the 5′ cap. Such a scenario has been demonstrated for rotaviral mRNAs, with the bridging interaction mediated by the viral protein NSP3 (49). For the DEN 3′ UTR, however, the synergy we have observed does not involve a viral protein, since the only viral protein present in our assays is a fragment of the capsid protein.

The quite significant difference in synergy observed with globin or DEN 5′ UTRs is an interesting observation. Both the GCLD and DCLD RNAs used in our synergy studies possess

the cCS1 and CS1 elements and therefore have the potential to cyclize through long-distance hybridization. Although this annealing would be disrupted by elongating ribosomes passing through cCS1, it may influence the initiation of translation. By forming a closed loop through direct RNA-RNA interaction rather than though protein mediation, translation may potentially be enhanced. This type of effect has been observed with *Barley yellow dwarf virus* RNA through direct RNA interaction between 5′ and 3′ UTRs (15). Alternatively, cCS1/CS1 interaction may be inhibitory towards translational initiation. This is suggested by the level of LUC expression from DCLD RNA being lower than that from DLD (Fig. 3) or DCLGpA RNA (Fig. 4), which contain only CS1 or cCS1. Recent modeling of the RNA folding of flaviviral RNAs has suggested the existence of RNA pairing between part of the 5′ UTR just upstream of the AUG initiation codon and the 3′ UTR (48). For DCLD RNA but not GCLD RNA, this pairing would occur in addition to the cCS1/CS1 interaction. Further experiments are needed to investigate whether these combined interactions are responsible for the cap/3′ UTR synergy observed for DCLD RNA being lower than that for GCLD RNA and also for the translational expression level from DCLD RNA being lower than that from DLD RNA (Fig. 3).

Complementarity between cCS1 and CS1 is not necessary for efficient translation driven by DEN UTRs. The speculation above explored a possible inhibitory interaction between 5′ and 3′ genomic sequences that has yet to be tested experimentally. We did, however, test the opposite effect, that is, whether cCS1/CS1 complementarity and these sequences themselves are required for efficient translation. Replacement of cCS1 with a modified version of CS1 had little influence on translational expression (Fig. 5C, *DCmLD). By contrast, replacement of CS1 decreased translational efficiency to about one-fifth (Fig. 5C, *DCLDm), and there was a further reduction when these mutations were combined to permit potential hybridization and RNA cyclization (Fig. 5C, *DCmLDm). These results indicate that cCS1/CS1 complementarity is not an attribute that supports translation. Indeed, the marginally increased translational efficiency of DCmLD RNA over DCLD RNA and the lower efficiency of DCmLDm DNA relative to DCLDm RNA support the idea that the long-distance RNA-RNA interactions interfere with translation. Such long-distance interactions have been implicated in flaviviral RNA replication (6, 21, 30, 50, 51), and it is intriguing to consider that this interaction may play a role in regulating the transition from the translation to the replication phase of the infection. If the cCS1/CS1 interaction can be stabilized, for instance, by the influence of bound proteins, this interaction might simultaneously inhibit translation and enhance replication. We have recently described an analogous switch involving translation factor eEF1A binding to the 3′-terminal region of *Turnip yellow mosaic virus* RNA (33, 34).

The low translational expression from DCLDm RNA suggests an independent role for CS1 in supporting translation. Inhibition of such a role may help to explain the potent inhibition of DEN replication by an antisense molecule directed at CS1 (24). No such role in translation was revealed by the only previous study of conserved sequence involvement in translational expression, performed using a WNV reporting replicon (30). In that study, neither the mutation of cCS1 nor that of CS1 detectably influenced the yield of the LUC reporter protein during the initial phase of viral gene expression that was not dependent on RNA replication. Further studies involving a variety of CS1 sequence variants and a comparison of the DEN and WNV systems are needed to clarify the situation. There may also be sensitivity differences between the replicon assay and the mRNA reporter assays we have used.

Multiple features of the DEN 3′ UTR enhance translational expression. CS1 is only one of a number of conserved elements present in the 3′ UTRs of flaviviral genomes (Fig. 1). By deleting each of the other elements (SLA, SLB, DB1, and DB2) from DCLD RNA, we tested their contributions to the stimulatory effect of the DEN 3′ UTR. Our results suggest, for the first time, that both RNA stability and translational enhancement are conferred by multiple features (Fig. 7). At this point, we cannot be certain that lost function is directly due to the absence of the deleted element. Indirect effects due to structural rearrangements in the deleted 3′ UTRs could have occurred, even though folding predictions suggested that this was not the case. Using a reporting replicon, Lo et al. (30) recently reported that each of the conserved sequence elements in the 3′ UTR of WNV RNA could be mutated or deleted without noticeably affecting translational expression in BHK cells. As noted above, these differences between the DEN reporter RNA and WNV replicon systems will need to be explored.

Among the features that contributed to fully efficient translation were the conserved 3′-terminal stems SLA and SLB. The deletion of either of these decreased translational efficiency to 40 to 50% of that of DCLD RNA (Fig. 7). Holden and Harris (18) have also recently observed that simultaneous deletions of DEN2 SLA and SLB ("3′SL" in their nomenclature) resulted in a similar decrease in translational expression in BHK cells from a reporter RNA like ours. In contrast, Brinton and colleagues have viewed SLA and SLB as negative regulators of translation in the WNV system. That conclusion arose from the observation that addition of WNV SLA and SLB to the 3′ UTRs of reporter mRNAs suppressed translation considerably in vitro, though only marginally in BHK cells (28). Further investigation will be needed to explain these differences.

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